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Identification of Genes Involved in Replication and Movement of Peanut Clump Virus

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The genome of peanut clump pecluvirus (PCV) consists of two messenger RNA components which contain, respectively, three and five open reading frames (ORFs). Inoculation of transcripts from full-length cDNA clones derived from the PCV RNAs showed that RNA-1 is able to replicate in the absence of RNA-2 in protoplasts, but both RNAs are necessary for plant infection. To investigate the role of different gene products in viral RNA replication and movement, transcripts from mutant cDNA clones were inoculated to protoplasts and to *Chenopodium quinoa* or *Nicotiana benthamiana* plants, and progeny RNA was detected by Northern blot analysis. The protein P15, encoded by the third ORF of RNA-1, is essential for efficient replication of the viral genome. The three proteins, P51, P14, and P17, of the triple gene block contained in RNA-2 are involved in localized movement of the viral genome, whereas the coat protein (P23) is also required for vascular movement. Insertion of the β-glucuronidase reporter gene (GUS) in place of the P23 or P39 genes (the first and the second genes of RNA-2) allows visualization of the virus infection in inoculated leaves. Although the presence of the GUS gene resulted in a lower accumulation of progeny RNA and, despite instability of the construct *in planta*, histochemical detection of PCV multiplication was more sensitive than Northern blot detection.

Key Words: pecluvirus; RNA replication; virus movement; GUS; plant virus.

INTRODUCTION

Peanut clump virus (PCV) causes serious diseases in peanuts in West Africa. The virions are rod-shaped and are transmitted by the plasmodiophoromycete fungus, *Polymyxa graminis*, leading PCV to be initially classified in the furoviruses genus (Thouvenel and Fauquet, 1981). Recently, PCV was reclassified as type member of the newly formed pecluvirus genus (Pringle, 1997; Torrance and Mayo, 1997) because its genome organisation differs significantly from soil-borne wheat mosaic furovirus (SBWMV), the type member of the furoviruses.

The bipartite genome of PCV comprises RNA-1 of 5897 nt and RNA-2 of 4504 nt (Fig. 1). RNA-1 contains three ORFs corresponding to P131, P191, and P15. P131 and P191 contain motifs characteristic of proteins with methyltransferase and helicase activity, and P191 contains motifs characteristic of an RNA-dependent RNA polymerase (Herzog, 1996). P15, which is expressed via a subgenomic RNA, displays homology with a protein containing conserved cysteine residues located at the 3′ ends of barley stripe mosaic hordeivirus (BSMV) γRNA, of poa semilatent hordeivirus (PSLV) γRNA and of SBWMV RNA-2. In the case of BSMV, this protein has been shown to be involved in the regulation of viral gene expression (Petty *et al.,* 1990; Donald and Jackson, 1994).

PCV RNA-2 encodes five ORFs (Herzog *et al.,* 1994). The 5′ proximal ORF corresponds to the coat protein (P23 or CP) and the next ORF to a protein of 39 kDa (P39), expressed by leaky scanning mechanism. This ORF is extensively deleted in some PCV isolates (Manohar *et al.*, 1993) which systemically infect *Nicotiana benthamiana,* suggesting that P39 may be involved in the transmission by the fungus. The three 3′ proximal ORFs encode the proteins P51, P14, and P17, respectively, which are similar to the triple gene block (TGB) movement proteins found in beet necrotic yellow vein virus (BNYVV), BSMV, potexviruses, and carlaviruses (Manohar *et al.,* 1993).

PCV resembles SBWMV and BSMV when replicase genes are compared but differs from SBWMV with respect to the movement protein. Sequence comparisons between their CP and movement proteins indicates that PCV is relatively closely related to BSMV (Herzog *et al.*, 1994; Wesley *et al.,* 1994).

In the present study, we have prepared *in vitro* transcripts derived from wild-type and mutant PCV cDNA clones to assess the requirement for the different gene products in RNA replication and movement for short and long distances. We also show that RNA-2 transcripts into which the β-glucuronidase gene (GUS) has been in-

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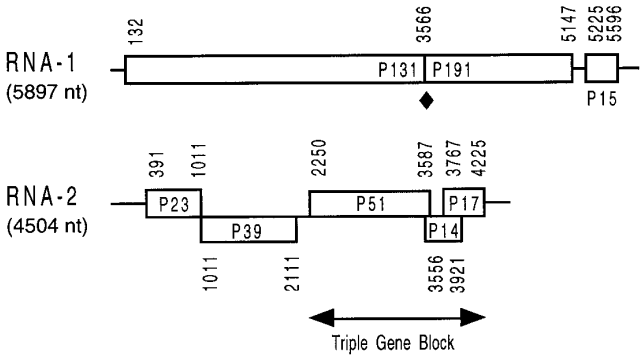

FIG. 1. Organization of PCV genome. ORFs are indicated by rectangles. The numbers correspond to the first and the last nucleotide of the different ORFs. ♦, suppressible termination codon.

serted can be used as a reporter for easy detection of the biological activity of the transcripts.

RESULTS

Infectivity of the transcripts TPC1 and TPC2

To determine whether *in vitro*-transcribed PCV RNAs behave like the natural viral RNAs, their capacity to replicate in protoplasts, to produce a local infection in inoculated *Chenopodium quinoa* leaves, and to establish a systemic infection in *N. benthamiana* was tested. Northern blot analysis of RNA from protoplasts of *C. quinoa* extracted at different times after infection by TPC1 and TPC2 showed that newly synthesized RNA-1 and -2 became detectable 12 h postinoculation (h p.i.) and continued to accumulate until 48 h p.i., reaching amounts comparable to those observed in protoplasts infected with viral RNA (Fig. 2). TPC1 was able to replicate in the absence of TPC2 (Fig. 2) while TPC2 did not replicate alone (not shown), confirming that RNA-1 encodes the genes involved in the replication of both RNA-1 and -2.

Ten days after inoculation of TPC1 and TPC2 to *C. quinoa,* faint chlorotic local lesions similar to those obtained within 5 days on leaves infected with viral RNA were observed. However, Northern blot analysis revealed that RNA-1 and -2 had been synthesized and encapsidated (Fig. 3 and Table 1).

In inoculated *N. benthamiana* leaves, viral RNA was readily detectable at 11 days p.i. (d p.i.), but only a faint signal of viral RNA was observed with extraction protocol B, in which the crude tissue extract was incubated at 37°C for 30 min prior to phenol extraction. We conclude that, at most, only an estimated 2–4% of the viral RNA was present in encapsidated form in the sample (Fig. 3 and Table 1). Symptoms consisting of chlorotic zones and stunting of apical leaves similar to those produced in viral RNA infected plants appeared between 7 and 10 d p.i. In these leaves, RNA-1 and -2 accumulated and were encapsidated in amounts similar to those obtained in virus-infected plants (Fig. 3 and Table 1).

Replication of TPC1 and TPC2 mutants in protoplasts

The two first genes of RNA-1 contain consensus sequences characteristic of viral RNA replicases (Koonin, 1991; Koonin and Dolja, 1993). To investigate the role of the third gene on RNA-1, we prepared mutant TPC1-15Nh, which encodes a truncated P15. When this mutant was coinoculated with TPC2 to protoplasts, accumulation of both RNA-1 and RNA-2 was very low (Fig. 4). *A priori,* it cannot be excluded that the mutation produced a conformational change of RNA-1 which could result in decreased affinity of the replication complex for the RNA. However, preliminary experiments have revealed that the replication level of viral RNAs was recovered by addition in the inoculum of a truncated RNA-expressing P15 (data not shown), indicating that P15 plays a role in viral RNA replication.

Mutants of RNA-2 which do not express the first gene [TPC2-CPAUG(−) and TPC2-CPFS] or the second gene [TPC2-39AUG(−) and TPC2Δ1] or one of the TGB genes [TPC2-51AUG(−), TPC2-14AUG(−), and TPC2-17-AUG(−)] were all able to replicate in protoplasts with nearly the same efficiency as wild-type TPC2 (Fig. 5 and Table 1).

Multiplication of TPC2 mutants in plants (Table 1)

Since none of the proteins encoded by RNA-2 is necessary for the replication of the RNA, we have investigated the role of these proteins in establishing infection of the two host plants.

Viral RNA accumulated in *C. quinoa* inoculated leaves

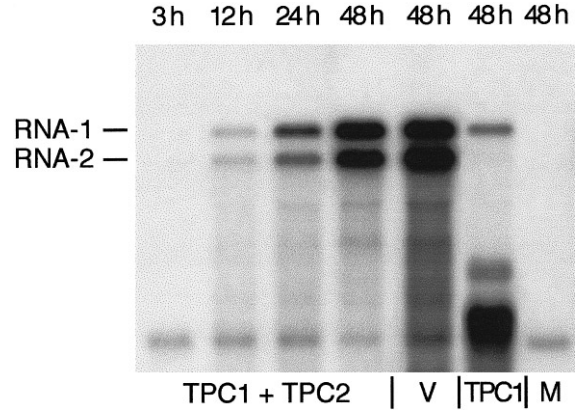

FIG. 2. Replication of TPC1 and TPC2 transcripts in *C. quinoa* protoplasts. Northern blot analysis of total RNA extracted from *C. quinoa* protoplasts inoculated with TPC1 and TPC2, viral RNA (V), TPC1, or mock inoculated (M) and harvested at the indicated time postinoculation. RNA-1 and RNA-2 were detected by hybridization with a riboprobe complementary to nucleotides 5193–5515 for RNA-1 and a riboprobe complementary to nucleotides 1138–4024 for RNA-2.

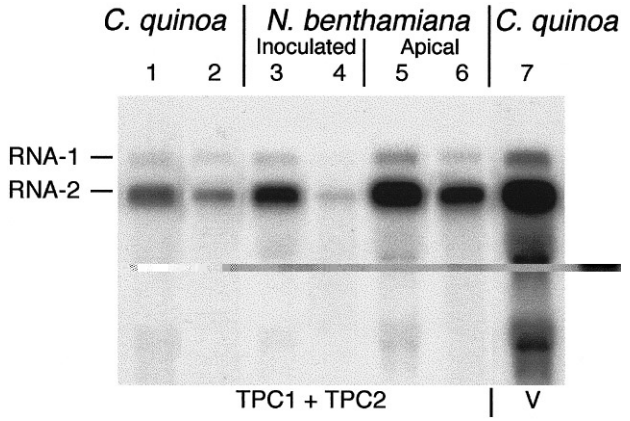

FIG. 3. Northern blot analysis showing accumulation of PCV RNAs in *C. quinoa* and in *N. benthamiana* leaves. Total RNA (lanes 1, 3, 5, and 7) or encapsidated RNA (lanes 2, 4, and 6) extracted from inoculated *C. quinoa* leaves (lanes 1 and 2), inoculated *N. benthamiana* leaves (lanes 3 and 4), or apical *N. benthamiana* leaves (lanes 5 and 6) infected with TPC1 and TPC2 and total RNA extracted from *C. quinoa* leaves inoculated with viral RNA (lane 7) were analyzed 11 d p.i. by Northern blot, using the riboprobe complementary to nucleotides 5193–5515 for RNA-1 and a riboprobe complementary to nucleotides 1–393 for RNA-2. Exposure of lane 4 was 10 times longer than that of other lanes.

infected with CP-defective mutants [TPC2-CPAUG(−), TPC2-CPFs]; but no viral RNA was detected in apical leaves of *N. benthamiana* 11 d p.i. (Table 1). Thus the CP is required for spread of the virus over long distances. When mutant transcripts TPC2-51AUG(−), TPC2-14AUG(−), and TPC2-17AUG(−), which do not express one or the other of the proteins of the TGB, were inoculated, progeny RNA was not only absent in apical leaves of *N. benthamiana* but also in inoculated *C. quinoa* leaves, confirming that, as for other viruses with a TGB, the three proteins are essential for cell-to-cell movement (Petty and Jackson, 1990; Beck *et al.*, 1991; Gilmer *et al.,* 1992). Mutants TPC2-39AUG(−) and TPC2Δ1, on the other hand, were amplified similarly to TPC2 in inoculated *C. quinoa* leaves or in apical *N. benthamiana* leaves, although the absence of P39 seems to slightly decrease the percentage of encapsidated RNA detected in apical *N. benthamiana* leaves. Therefore, neither the nucleotide sequence nor the protein itself (P39) were strictly necessary to promote amplification of RNA and movement of the virus.

Replication capacity of RNA-2 transcripts containing the GUS gene

Since null mutations of the P39 and the CP have different effects in virus movement, we tested the possibility of substituting the reporter gene GUS for these genes to easily follow the spread of the virus in *C. quinoa* (the hypersensitive host plant) or in *N. benthamiana* (the systemic host plant) plants.

Transcripts TGUS/CP, TGUS/39, and the corresponding transcripts deleted from the TGB (TGUS/CPΔTGB and TGUS/39ΔTGB) were all able to replicate in protoplasts although the presence of the GUS gene significantly decreased the accumulation of progeny RNA, especially for the nondeleted mutants (Fig. 7A). The GUS activity measured by fluorometric assay was about five times higher in extracts of protoplasts infected with TGUS/CP as in protoplasts infected with TGUS/39. This difference can be correlated to the relative expression levels of CP and P39 observed by *in vitro* translation studies (Herzog *et al.,* 1995).

Visualization of GUS expression and multiplication of transcripts

The presence of GUS activity in *C. quinoa* and *N. benthamiana* inoculated leaves was detected by the histochemical assay. At 3 d p.i., intense blue spots were visualized on *C. quinoa* leaves inoculated with TGUS/CP

TABLE 1

					N. benthamiana					
		C. quinoa			Inoculated leaves			Apical leaves		
Inoculum	Protoplasts	T	E	% E/T	T	E	% E/T	T	E	% E/T
TPC1 + TPC2	+	+	+	40–60	+	+	2–4	+	+	40–60
TPC1 + TPC2-CPAUG(−)	+	+	NT		NT	NT		−	NT	
TPC1 + TPC2-CPFS	+	+	NT		NT	NT		−	NT	
TPC1 + TPC2-51AUG(−)	+	−	NT		NT	NT		−	NT	
TPC1 + TPC2-14AUG(−)	+	−	NT		NT	NT		−	NT	
TPC1 + TPC2-17AUG(−)	+	−	NT		NT	NT		−	NT	
TPC1 + TPC2-39AUG(−)	+	+	+	40–60	+	+	1–2	+	+	20–30
TPC1 + TPC2Δ1	+	+	+	40–60	+	+	1–2	+	+	20–30

Note. Presence (+) or absence (−) of detectable RNA-2 at 11 d p.i. by Northern blot hybridization using an RNA-2 specific riboprobe. The proportion of encapsidated RNA (extracted by protocol B) to total RNA (extracted by protocol A) was calculated by comparison of autoradiography bands of similar intensity obtained after different exposure times. T; total RNA; E; encapsidated RNA; NT; not tested.

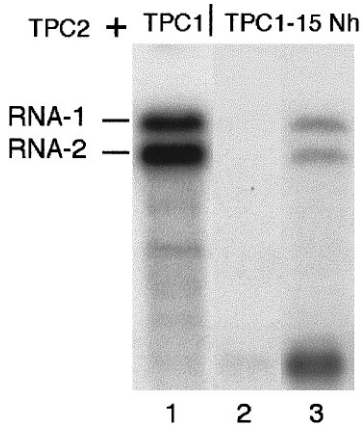

FIG. 4. Replication of the mutant TPC1-15Nh in protoplasts. *C. quinoa* protoplasts were inoculated with TPC2 plus TPC1 (lane 1) or plus mutant TPC1-15Nh (lanes 2 and 3) and harvested at 48 h pi. The extracted RNA was subjected to Northern blot analysis as described in Fig. 2. Exposure to X-ray film was 10 times longer for lane 3 than for lanes 1 and 2.

(Fig. 6A) and a few pale blue spots were observed on those infected with TGUS/39 (Fig. 6B). Later on (7 d p.i.), the spots were of similar intensity in both cases (Figs. 6C and 6D). However, GUS activity measured by fluorimetric assay was about 10 times higher in extracts of TGUS/CP *C. quinoa*-inoculated leaves compared to those of TGUS/39 at 3 d p.i. and 40 times higher at 11 d p.i. Northern blot analysis performed at 11 d p.i. showed that similar amounts of progeny RNA had accumulated in leaves infected with the two types of transcripts (Fig. 7B, lanes 2 and 4). In the progeny of TGUS/39 infections, however, the presence of a smear corresponding to shorter RNA species revealed the occurrence of deletion within the RNA which may account for a loss of GUS gene and the loss of GUS activity. Only very rare pinpoint blue spots were observed on *C. quinoa* leaves infected with TGUS/CPΔTGB or TGUS/39ΔTGB (not shown), and Northern blot analysis revealed that RNA progeny was not detectable in these leaves (Fig. 7B, lanes 3 and 5). Thus histochemical analysis of GUS activity in *C. quinoa* leaves is a faithful reflection of the competence of the various mutants to move from cell to cell. It should be noted that the proportion of encapsidated RNA to total progeny RNA recovered from TGUS/39-infected *C. quinoa* was significantly lower than from TPC2-infected plants (Fig. 7B, lanes 6 and 8), suggesting that the GUS gene hinders encapsidation of the RNA molecule.

The histochemical image of GUS activity in inoculated leaves of TGUS/CP- and TGUS/39-infected *N. benthamiana* were quite different. While blue spots were clearly detected at 3 d p.i. for TGUS/CP (Fig. 6E), no spots were visualized for TGUS/39 (Fig. 6F). At 7 d p.i., the spots had expanded in the first case while only a few very pale blue large spots were detected for TGUS/39. At 11 d p.i., the spots continued to expand slightly, but some of them were blue only at the periphery (Figs. 6G and 6H). On leaves infected with TGB null mutants, pinpoint spots indicated that only a subliminal infection had occurred and that there was little if any movement from cell to cell. Microscopic observations performed at 11 d p.i. revealed that these spots contained between 1 and 5 cells (Fig. 6I), whereas spots observed at 3 d p.i. on TGUS/CP infected leaves contained about 50 cells (Fig. 6J). In apical leaves of TGUS/CP-infected *N. benthamiana*, no GUS activity was detectable, which is consistent with the absence of symptoms on those plants and with the requirement of CP for long-distance movement of the virus. But surprisingly, GUS activity was also not detectable in apical leaves of TGUS/39-infected *N. benthamiana* although intense symptoms developed. Northern blot analysis of total RNA extracted from inoculated leaves of *N. benthamiana* at 11 d p.i. revealed that no progeny RNA was detected in leaves inoculated with TGUS/CPΔTGB and TGUS/39ΔTGB (Fig. 7C, lanes 3 and 5) and that progeny RNA-2 was detected in TGUS/CP- and TGUS/39-infected leaves (Fig. 7C, lanes 2 and 4); but most of this RNA was significantly shorter than the inoculated transcript and probably corresponds to molecules in which the GUS gene was partially or completely deleted. In both cases, no encapsidated viral RNA was detectable (not shown). In apical leaves of *N. benthamiana* inoculated with TGUS/CP, TGUS/CPΔTGB, and TGUS/39ΔTGB, progeny RNA was absent (Fig. 7C, lanes 7, 8, and 10). In apical leaves of TGUS/39-inoculated *N. benthamiana,* both free and encapsidated progeny RNA was present, but most molecules were of the size of TPC2Δ1 (not shown) and presumably had undergone deletion of the GUS gene, which would explain the absence of GUS activity in these leaves. In some experiments, progeny RNA was absent in apical leaves of TGUS/39-inoculated *N. benthamiana*. In these cases, no

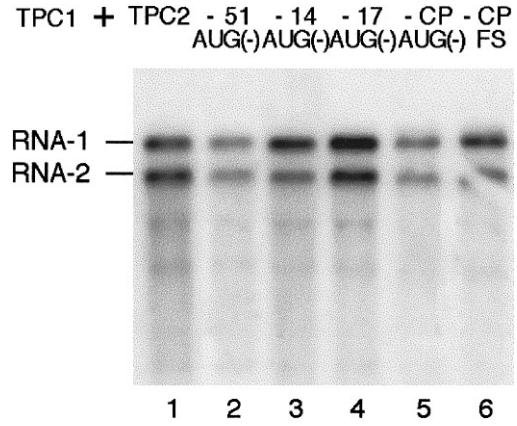

FIG. 5. Replication capacity of TPC2 mutants in *C. quinoa* protoplasts. Protoplasts were infected with TPC1 and TPC2 (lane 1), or TPC1 and TPC2-51AUG(−) (lane 2), TPC2-14AUG(−) (lane 3), TPC2-17AUG(−) (lane 4), TPC2-CPAUG(−) (lane 5), or TPC2-CPFS (lane 6), and harvested 48 h pi. The extracted RNA was subjected to Northern blot analysis as in Fig. 2.

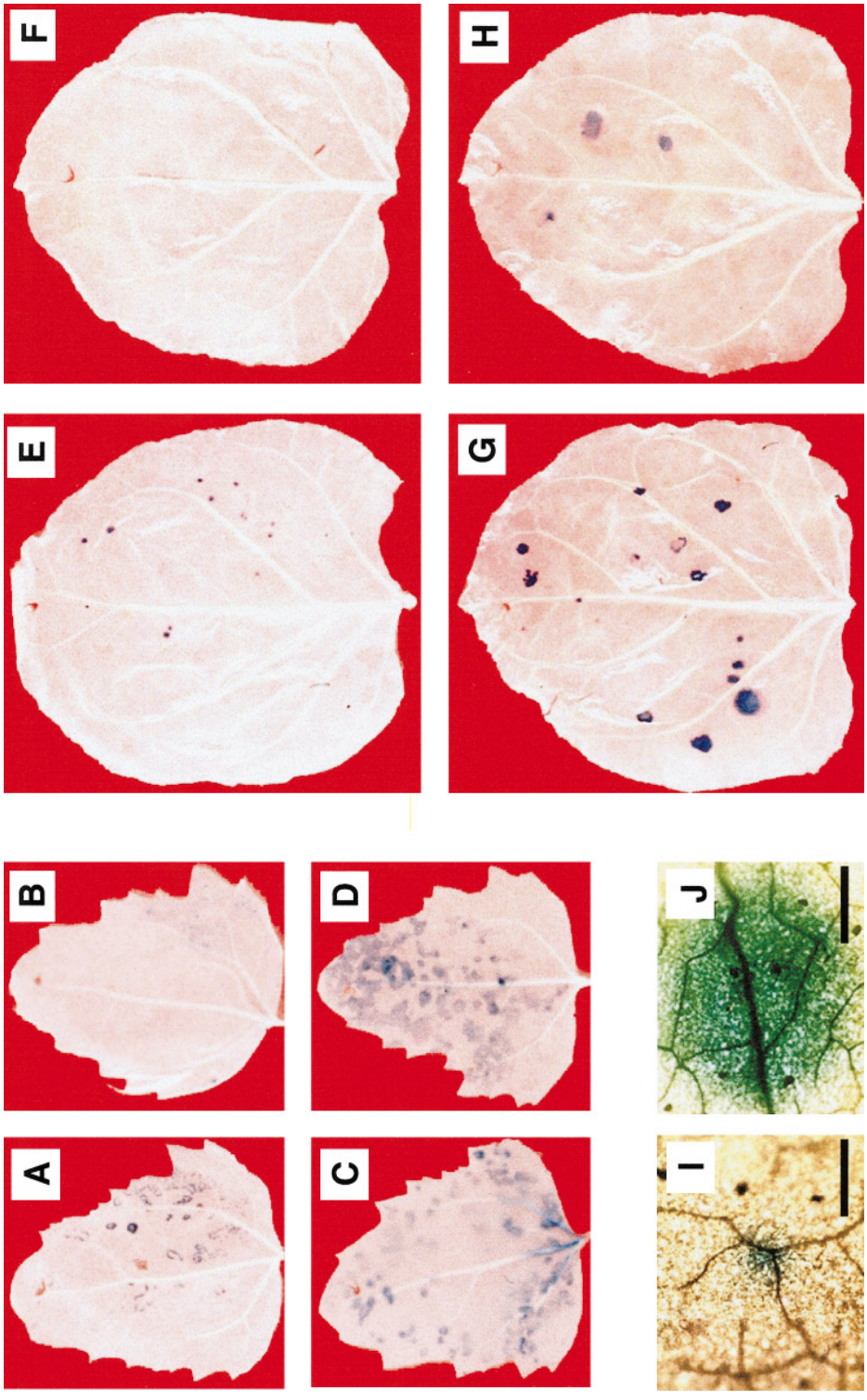

FIG. 6. Histochemical analysis of GUS activity in inoculated leaves of *C. quinoa* (A–D) and *N. benthamiana* (E–J). The photographs are macroscopic (A–H) and microscopic (I and J) images of infiltrated leaves following inoculation with TPC1 and TGUS/CP (A, C, E, G, and J) or/and TGUS/39 (B, D, F, and H) or/and TGUS/CP△TGB (I). Observations were performed 3 days (A, B, E, F, and J), 7 days (C and D) or 11 days (G, H, and I) postinoculation. Bar represents 100 μm.

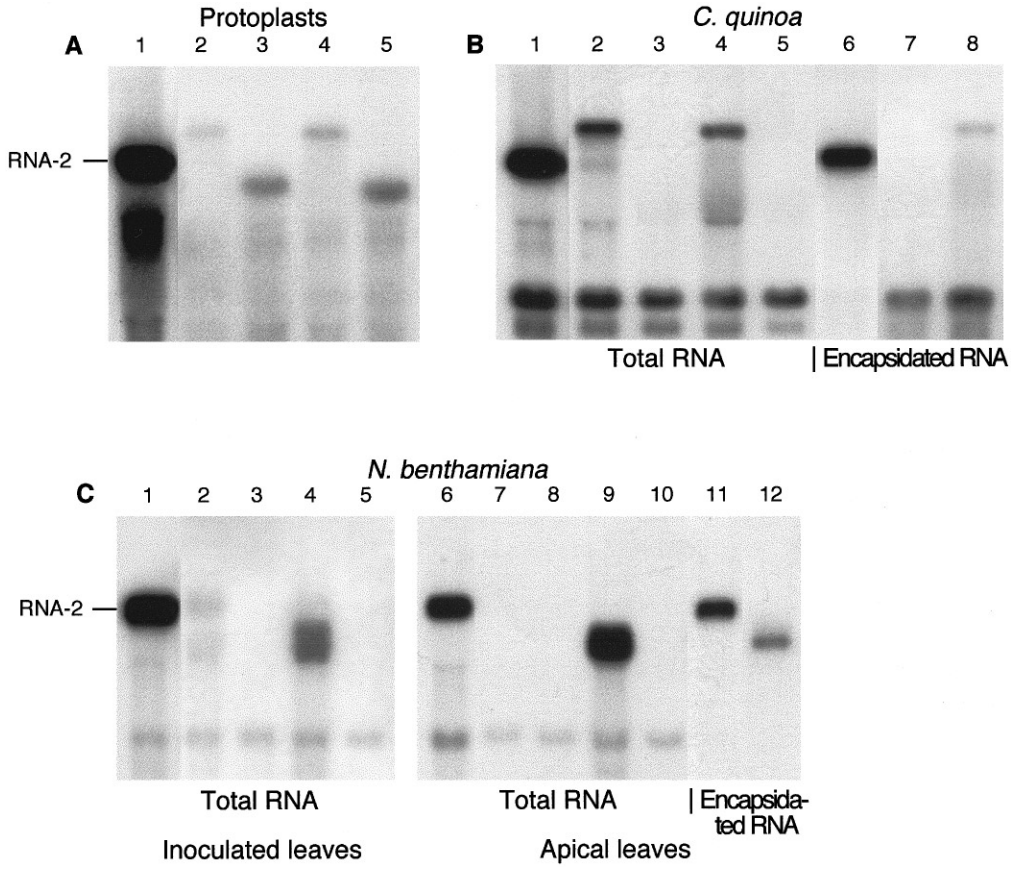

FIG. 7. Northern blot analysis of free and encapsidated PCV RNA-2 extracted from protoplasts and plants infected with TPC1 and TPC2/GUS mutants. (A) RNA extracted from protoplasts of BY-2 cells at 48 h p.i. with TPC1 and TPC2 (lane 1) or TPC1 and TGUS/CP (lane 2), TGUS/CP△TGB (lane 3), TGUS/39 (lane 4), or TGUS/39△TGB (lane 5). (B) Total RNA (lanes 1–5) or encapsidated RNA (lanes 6–8) extracted 11 d p.i. from inoculated leaves of *C. quinoa* infected with TPC1 and TPC2 (lanes 1 and 6) or TPC1 and TGUS/CP (lanes 2 and 7), TGUS/CP△TGB (lane 3), TGUS/39 (lanes 4 and 8), or TGUS/39△TGB (lane 5). (C) Total RNA (lanes 1–10) or encapsidated RNA (lanes 11 and 12) extracted 11 d p.i. from inoculated leaves (lanes 1–5) or apical leaves (lanes 6–12) of *N. benthamiana* infected with TPC1 and TPC2 (lanes 1, 6, and 11) or TPC1 and TGUS/CP (lanes 2 and 7), TGUS△TGB (lanes 3 and 8), TGUS/39 (lanes 4, 9, and 12), or TGUS/39△TGB (lanes 5 and 10). PCV RNA-2 was revealed using a riboprobe complementary to nucleotides 1–393 of RNA-2. Exposure of lanes 7B and 8B and exposure of lanes 6–12C were, respectively, 10 times longer and 4 times shorter than that of the other lanes.

deletion of the progeny RNA-2 extracted from inoculated *C. quinoa* and *N. benthamiana* leaves was visible. Thus it appears that only progeny RNA-2 molecules from which the GUS gene has been partially or completely ejected are encapsidated and therefore can migrate to apical leaves.

DISCUSSION

Our results show that the third ORF of PCV RNA-1, encoding the protein P15, is essential for efficient replication of the viral genome. When compared with the small cysteine rich proteins (CRP) encoded by the 3′ terminal region of hordei-, furo-, tobra-, and carlaviruses, the best homologies were found to be with BSMV γb, SBWMV P19, and PSLV P19 (Herzog *et al.,* 1994). But whereas for these latter proteins, the cysteine and histidine residues are organized in two clusters, only the second cluster was present in PCV P15. It has been shown that BSMV γb, and BNYVV P14 play an important role in the accumulation of other viral proteins, essentially of CP and movement proteins (Petty *et al.,* 1990; Donald and Jackson, 1994; Hehn *et al.,* 1995). However, the mechanisms involved are still unknown. Koonin *et al.* (1991) suggested that the CRP proteins might be involved in the transactivation of the viral RNA synthesis, and Donald and Jackson (1996) proposed the hypothesis that regulatory properties of BSMV γb protein could be mediated, in part, by its RNA binding activities. Similarly, P15 may be involved in the activation of the replication or of the translation of RNA-1, but it could also act indirectly by targeting the viral RNAs to their replication site.

No RNA amplification was observed in inoculated *C. quinoa* or *N. benthamiana* leaves with any of the mutant transcripts which do not express one of the TGB proteins P51, P14, or P17. Thus for PCV, as for all other viruses containing a TGB, all three proteins are individually re-

quired for localized (cell-to-cell) movement. The CP is not needed in this step as CP-deleted mutants were amplified in inoculated *C. quinoa* and in *N. benthamiana* leaves. But these mutants do not accumulate in upper uninoculated leaves of *N. benthamiana,* suggesting that CP is needed for vascular movement of PCV. Many plant viruses which move through the phloem encode proteins that provide functions needed for this transport (Carrington *et al.,* 1996). In the case of TMV, Ding *et al.* (1992) showed that MP fails to dilate plasmodesmata between bundle sheath cells and phloem parenchyma cells, whereas Cronin *at al.* (1995) suggested that defective long-distance movement was associated with impeded entry into or exit from sieve elements. In both cases, long-distance movement requires the involvement of a supplementary protein not needed for cell-to-cell movement, i.e., the CP in the case of TMV (Ding *et al.,* 1996) and helper component proteinase in the case of TEV. Most viruses containing a TGB require CP to move long distances, although this protein is not required for BSMV systemic infection (Petty *et al.,* 1990), whereas it is already needed for the cell-to-cell movement of PVX (Baulcombe *et al.,* 1995). The involvement of CP for long-distance movement in most cases seems to be linked to its role in packaging the RNA (Seron and Haenni, 1996), but it is also possible that a ribonucleoprotein complex which includes the CP but is distinct from the virion is involved in vascular movement.

Encapsidation of PCV RNA occurs at very different levels in the three types of infected leaves analyzed in this work. In inoculated leaves of *C. quinoa*, about 40–60% of the synthesized viral RNA was encapsidated, whereas only about 2–4% was encapsidated in inoculated leaves of *N. benthamiana*. It is possible that encapsidation of the RNA molecules in a cell occurs only when a certain amount of progeny viral RNA has accumulated and/or when enough CP is produced. In *C. quinoa,* RNA movement is restricted to cells near the initial sites of infection, whereas in *N. benthamiana,* the infection invades the entire leaf. The concentration of RNA per infected cell is therefore probably much higher in inoculated leaves of *C. quinoa* than of *N. benthamiana*, although similar amounts of RNA have accumulated per gram of tissue. We suggest that, in the inoculated leaves of the systemic host, encapsidation occurs mainly in the cells in which the RNA has not exited before enough CP is synthesized to trigger encapsidation, i.e., in phloem parenchyma cells and/or bundle sheath cells. In mesophyll cells, because RNA moves from cell to cell, the net amounts of RNA and CP would increase only slowly, so that levels sufficient for virion formation are only seldom attained. Indeed the leaf may become senescent before virions can accumulate. In apical *N. benthamiana* leaves, the amounts of accumulated RNA are much higher than in inoculated leaves, and newly synthesized RNA cannot move further so that the concentration of RNA per cell should rapidly attain a level sufficient for encapsidation.

Insertion of the GUS gene in place of the CP or P39 genes (both nonessential for short distance movement) allows the direct visualization of the virus infection in inoculated leaves of host plants, although the accumulation of transcripts containing the foreign gene was drastically reduced. This lower accumulation may be a consequence of the increased length of mutants containing the GUS gene, either because the transcripts are less stable or less efficiently replicated. However, mutants in which the green fluorescent protein (GFP) gene was inserted instead of the GUS gene were also inefficiently replicated, although their length was similar to that of wild-type RNA-2. It is likely that the small proportion of encapsidated progeny RNA of TGUS/39 detected in *C. quinoa* leaves (in which about 40–60% of the wild-type TPC2 progeny was encapsidated) is a consequence of the poor replication efficiency of the transcripts if, as we suggested above, encapsidation occurs only when sufficient RNA and CP have accumulated.

Despite the low replication efficiency of TGUS/CP, the GUS histochemical assay was more sensitive than Northern blot detection. Indeed, subliminal infections with transcripts containing deletions in the TGB were visible. Also blue spots can be clearly visualized on inoculated *N. benthamiana* leaves at 3 d p.i. whereas progeny RNA cannot be detected by Northern blot analysis. Moreover, at 11 d p.i., spots were still visualized, although only a few molecules, detected after a long autoradiographic exposure, remain complete. Therefore TGUS/CP may be a useful tool to investigate the host range of PCV. Inoculation of TPC2GUS/39 to *N. benthamiana* did not allow us to follow the spread of the virus in apical leaves. As already observed in the case of TEV and PVX (Chapman *et al.,* 1992; Dolja *et al.,* 1992, 1993), longer periods of infection resulted in removal of the GUS gene and gave rise to deleted forms of the progeny RNA. Deleted forms appeared in the progeny of TGUS/CP and TGUS/39, but while excision of GUS appears to be complete in the latter case, it remains partial in the first case (the deleted molecules remaining longer than the wild-type TPC2). For PCV, strains exist in which the P39 gene is nearly completely removed, suggesting that rearrangement of the genome in this region may be better tolerated than elsewhere, not only because the gene is not essential but also because the stability of the molecule is less affected. Further studies on the stepwise rearrangement of the progeny of TGUS/CP and TGUS/39 should provide better knowledge of the factors involved in the rearrangement of the genome and perhaps help to construct more stable TPC2GUS transcripts.

MATERIALS AND METHODS

Virus and viral RNA

Purification of PCV (isolate PCV2) from systemically infected *N. benthamiana* leaves and extraction of viral RNA was as described (Manohar *et al*., 1993).

Host plants

PCV causes local lesions on the inoculated leaves of *C. quinoa* and systemically infects *N. benthamiana* producing chlorosis, deformation of apical leaves, and stunting of the plant. Infected plants were grown in a controlled environment chamber at 26°C with 16 h illumination.

Construction of recombinant plasmids

Full-length cDNA plasmids were prepared by stepwise ligation of different recombinant plasmids, containing overlapping fragments of RNA-1 or RNA-2 cDNA, after digestion at a unique restriction enzyme site present in the overlapping region.

The plasmids containing cDNA of RNA-1 were prepared as described (Herzog *et al.,* 1994), essentially by using synthetic oligomers, complementary to known portions of the RNA-1 sequence, to prime the first strand synthesis. The second strand was obtained by the method of Gubler and Hoffman (1983). The oligonucleotide ACAGCTGCAGATCGATACGCGTGGGACGGATATCGCTCCG used to prime the synthesis of cDNA corresponding to the 3′ end of RNA-1 contained 19 nucleotides complementary to the 3′ end of the RNA (underlined) and was flanked at its 5′ end by 21 nucleotides containing *Mlu*I and *Pst*I restriction sites.

The recombinant plasmid containing the 5′ end of RNA-1 was constructed by insertion of a cDNA fragment obtained by reverse transcription followed by the polymerase chain reaction (PCR); the oligonucleotide AGGGGTACCATGGTAATACGACTCACTATAGGTATTCTGTGGTGTTTGTT used to prime plus-strand synthesis contained a *Kpn*I restriction site, a T7 bacteriophage promoter, and 19 nucleotides of the 5′ end of the RNA (underlined). The full-length cDNA-1 obtained by ligation of five different recombinant plasmids was inserted between the *Kpn*I and *Pst*I restriction sites of pUC19 to obtain pPC1.

To construct full-length cDNA-2, the three recombinant plasmids pPC2–2223, pPC2–9, and pPC2–7 obtained by Manohar *et al.* (1993) were ligated and inserted in pUC18 between the *Bam*HI and *Hin*dIII restriction sites to produce pPC2a. The cDNA-2 insert in this construct contained 121 extranucleotides at its 3′ end mostly consisting of a poly-A sequence introduced by the cloning technique used. To eliminate these extra nucleotides, a PCR fragment of about 750 nucleotides was amplified between a primer corresponding to the sequence of RNA-2 from nucleotide 3763 to nucleotide 3783 and a primer which contained 18 nucleotides complementary to the 3′ RNA end from nucleotide 4486 to nucleotide 4504 and the sequence of the *Hin*dIII restriction site. After digestion by *Bgl*II and *Hin*dIII, the purified fragment was inserted in pPC2a in which the corresponding fragment was eliminated. The *Bam*HI-*Xho*I fragment corresponding to complete cDNA-2 was then inserted in pUC19 between the restriction sites *Bam*HI and *Hin*dIII to produce pPC2.

The sequence of all starting recombinant plasmids as well as the sequences around the restriction sites used for the ligation of successive plasmids were verified (Sanger *et al*., 1977).

Construction of mutants

Mutant pPC1–15Nh. Plasmid pPC1 was linearized by *Nhe*I digestion and religated after protruding ends were filled using the Klenow fragment of *Escherichia coli* DNA polymerase I. This resulted in the insertion of the four nucleotides CTAG at position 5513. Because of the frameshift, the translation termination of ORF3 occurred at the inserted UAG codon instead of at the UAA 5597, resulting in a truncated protein of 12 kDa.

Mutants pPC2-CPAUG(−) and pPC2-CPFS. For mutant pPC2-CPAUG(−), the initiation codon of the CP gene was replaced by AGA. This was achieved by replacing the *Bam*HI-*Nhe*I fragment in pPC2 (the sites of which are respectively located in the polylinker of the plasmid and at nucleotide 1620) with the equivalent fragment from plasmid p2MΔAUG (Herzog *et al*., 1995) containing the 5′ half of cDNA-2 which carries the mutation. Mutant pPC2-CPFS was constructed similarly by replacement of the *Bam*HI-*Nhe*I fragment of pPC2 with the equivalent fragment of mutant 2MUGA2 in which the insertion of four nucleotides between nucleotides 635 and 636 produced a frameshift in the CP ORF resulting in a protein of 9.3 kDa (Herzog *et al*., 1995).

Mutants pPC2-51AUG(−), pPC2-14AUG(−), pPC2-17AUG(−). Point mutations destroying the initiation codon of each ORF of the TGB were introduced in pPC2 by using overlap extension PCR (Ho *et al.,* 1989). For mutant pPC2-51AUG(−) the mutated fragment was from nucleotide 2001 to nucleotide 2948 and the ATG initiation codon of P51 was replaced by AAG. The resulting fragment was digested by *Bss*HII (nucleotide 2051) and *Eco*RI (nucleotide 2882) and substituted in pPC2 for the corresponding wild-type *Bss*HII-*Eco*RI fragment. To construct pPC2-14AUG(−), a fragment of 702 bp was amplified which extended from nucleotides 3390 to 4091, with the ATG of the P14 ORF replaced by ACG. The amplified fragment was digested by *Bst*XI (nucleotide 3492) and *Bgl*II (nucleotide 4021) and inserted into pPC2 in place of the wild-type fragment. Mutant pPC2-17AUG(−) was constructed in the same way as pPC2-14AUG(−) except that the ATG of P17 ORF was replaced by ACG. As the

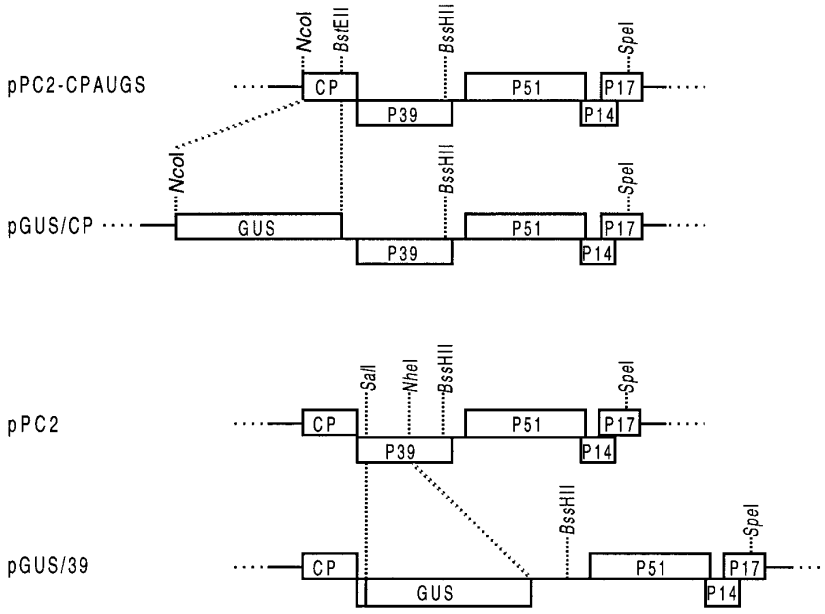

FIG. 8. Schematic representation of pGUS/CP and pGUS/39 obtained from pPC2-CPAUGS and pPC2, respectively, by insertion of GUS gene in place of CP or P39 genes.

TGB genes overlap, the two last mutations also produced nucleotide changes in the ORF of P51 or in the ORF of P14 but did not change the amino acid sequence of these proteins.

Mutants pPC2-39AUG(−) and pPC2Δ1. In mutant pPC2-39AUG(−), the initiation codon of the P39 cistron was replaced by UUG using overlap extension PCR. The PCR fragment containing the mutation was digested by *Bst*EII-*Sal*I (nucleotide 802 and nucleotide 1029) and introduced in pPC2 in place of the original fragment.

pPC2Δ1 corresponds to pPC2-39AUG(−) deleted between nucleotide 1034 to nucleotide 2051 and was obtained by digestion of pPC2-39AUG(−) with *Sal*I (nucleotide 1029) and *Bss*HII (nucleotide 2051) and recircularization after protruding extremities had been filled in using the Klenow fragment of *E. coli* DNA polymerase I.

Mutants pGUS/CP, pGUS/39, pGUS/CPΔTGB, and pGUS/39ΔTGB. In mutant pGUS/CP, the *E. coli* β-glucuronidase GUS gene (Jefferson *et al.,* 1986) was inserted in place of the CP gene in pPC2 (Fig. 8). pCaGa, kindly furnished by M. Lepetit (Jupin *et al.,* 1990), was digested by *Bam*HI, protruding ends were filled in, and the plasmid was then digested with *Nco*I. The fragment of 1861 nucleotides containing the GUS gene was inserted in pPC2-CPAUGS (Herzog *et al.,* 1995) in place of the *Nco*I-*Bst*EII fragment (nucleotides 389–802). pPC2-CPAUGS corresponds to pPC2 in which mutation of three nucleotides flanking the CP initiation codon created an *Nco*I restriction site.

For the mutant pGUS/39, the pCaGa *Nco*I-*Bam*HI fragment containing the GUS gene was filled in at both ends and then inserted in place of the *Sal*I-*Nhe*I (nucleotide 1029–1620) fragment of pPC2, in which protruding extremities had been filled in. In this case, GUS was expressed from the initiation codon of the P39 cistron and fused at its N terminus to eight amino acids of the N terminus of P39.

The two mutants pGUS/CPΔTGB and pGUS/39ΔTGB derived from pGUS/CP and pGUS/39 in which the *Bss*HII-*Spe*I fragment (from nucleotides 2051 to 4142) was removed.

Synthesis of transcripts

Capped *in vitro* transcripts were synthesized using T7 RNA polymerase from *Mlu*I-linearized wild-type or mutant pPC1 or from *Hin*dIII-linearized wild-type or mutant pPC2 as described by Herzog *et al.* (1995). In most experiments, the transcription kit of Promega was used to obtain high transcript yields. The resulting transcripts TPC1 and TPC2 contained only one extra G at the 5′ end and four extra nucleotides at the 3′ end.

Inoculation and analysis of plants and protoplasts

Protoplasts were prepared from *C. quinoa* leaves (Hemmer *et al.,* 1993) and from tobacco BY-2 cells (Nagata *et al.*, 1992) as described (Watanabe *et al.*, 1987) with slight modifications. An aliquot of 5-day-old cellular suspension was centrifuged at 800 rpm for 5 min. The pellet was resuspended in 2 volumes of 450 mM mannitol, pH 5.5; 700 μg/l MES (Medium A) containing 1% of Onozuka RS cellulase and 0.1% Y-23 pectolyase and maintained 2 h at 30°C in the dark with gentle agitation. Protoplasts were then collected by filtration through a 100-μm filter. The filtrate was centrifuged at 800 rpm and

after two washes in medium A, the protoplasts were resuspended in 450 mM mannitol, pH 5.5; 700 μg/l MES; 0.1 mM $CaCl_2$ at a concentration of about 2×10^6 protoplasts/ml.

Protoplasts (200,000) of *C. quinoa* or 1×10^6 protoplasts of BY-2 tobacco cells were infected by electroporation (450 V/cm, 10 W, 125 μF) with 5 μg each of transcript TPC1 and TPC2. Total nucleic acid was extracted from protoplasts 48 h p.i. as described by Hemmer *et al.* (1993).

C. quinoa or *N. benthamiana* plants were inoculated with 200 μl of 5 mM sodium phosphate, pH 7.5 containing 0.03% macaloid and 5 μg of each transcript. Generally, four leaves were inoculated (two leaves per plant). Inoculated and apical leaves of *N. benthamiana* were collected separately, whereas only inoculated leaves of *C. quinoa* were collected. The leaves were ground in liquid nitrogen and conserved at $-80°$C before RNA extraction.

Two procedures were used for RNA extraction from 200 μg of frozen powder of infected leaves. In protocol A, the powder was ground in 600 μl ice cold 200 mM Tris (pH 9), 400 mM KCl, 35 mM $MgCl_2$, 25 mM EGTA, and 200 mM sucrose, and total RNA was isolated by phenol extraction and ethanol precipitation. Control experiments in which purified PCV particles or RNA were added to healthy leaf tissue at the beginning of extraction have shown that both encapsidated and free viral RNA survive this procedure. In protocol B, the powder was homogenized in 600 μl of 50 mM Tris (pH 7.5), 10 mM $MgCl_2$, and the extract was incubated at 37°C for 30 min prior to phenol extraction. Control experiments showed that, by this procedure, encapsidated viral RNA is recovered, whereas naked RNA is degraded by the action of the nucleases present in the crude cell sap. Viral RNA was detected by Northern blot hybridization with *in vitro*-transcribed ^{32}P-labeled specific riboprobes.

Detection of β-glucuronidase activity

Histochemical analysis of GUS activity with the substrate 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) was performed as described (Jefferson, 1987). Leaves were infiltrated with substrate at 37°C overnight before clearing the tissue in ethanol.

Fluorometric GUS assays were carried out on 8 mg of tissue homogenized in 400 μl of GUS extraction buffer (Jefferson, 1987). Homogenate (300 μl) was added with 25 μl of assay buffer MUG (12.5 mM). The reaction was stopped immediately or after incubation for 45 or 90 min at 37°C by addition of 900 μl of stop buffer 0.2 M Na_2CO_3 to 100-μl aliquots. Fluorescence of samples was measured with a spectrofluorimeter (excitation at 365 nm, emission at 455 nm).

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